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OBJECTIVE: We are interested in identifying and characterizing the various immunoglobulin isotypes in cetaceans and in furthering our understanding of these molecules and their roles and functions within the cetacean humoral and mucosal immune systems. The goal of the work outlined in the proposal was identification and characterization of the various immunoglobulin isotypes in cetaceans and to further our understanding of these molecules and their roles and functions within the cetacean humoral immune system.

APPROACH:

Objective 1. Development of multiple heterohybridomas secreting the various classes of cetacean immunoglobulins: In efforts to accomplish objective one, we used standard techniques normally used for fusion of activated immune mouse lymphocytes with a standard mouse non-secreting myeloma cell line as fusion partner for production of mouse/mouse hybridomas secreting mouse monoclonal antibodies. By fusion with activated lymphocytes from the bottlenose dolphin (*Tursiops truncatus*), a dolphin/mouse "heterohybridoma" would be produced. Since neither cetacean splenic nor lymph node lymphocytes were available for use in the project, we employed peripheral blood lymphocytes from the bottlenose dolphin.

Objective 2. Production of mouse monoclonal antibodies specific for cetacean immunoglobulin isotypes: When sufficient amounts of each purified immunoglobulin isotype was collected, a set of five female BALB/c mice were immunized with the appropriate protein in preparation for the fusion protocol. Fusion was performed with the myeloma cell line CRL 1580 as the fusion partner with mouse spleen cells. Screening for secreting hybridomas was done by using an enzyme linked immunosorbent assay (ELISA). The contents of the wells containing viable cells and having tested positive for immunoglobulin production were removed for limiting dilution cloning. Once a population of clones was established, screening for the specificity of the antibody produced was begun. An ELISA designed to detect clones producing antibodies against one or more of the different classes of dolphin immunoglobulins was employed to demonstrate the specificity of the monoclonal antibody. Clones producing antibodies of potentially desirable specificity were expanded and frozen in liquid nitrogen for long term storage. Once a desired specificity was demonstrated by application of the ELISA's described above, immunostains were then performed to further clarify the specific reactivity of the monoclonal antibody for specific dolphin isotypes and to check for cross-reactivity with other proteins. To complete the initial characterization of a developmental monoclonal antibody, a mouse monoclonal antibody isotyping strip was then applied to a fresh dilution of hybridoma media was added to a development tube which contains colored latex beads coated with anti-mouse kappa and anti-mouse lambda antibodies which will bind any mouse antibodies if present. Based these results, affinity chromatography or other purification method can be performed to remove the monoclonal antibody from the hybridoma media.

Objective 3. Completion of the identification of the classes and subclasses of the immunoglobulins present in Tursiops truncatus: The IgG of Tursiops truncatus was purified by affinity chromatography using Protein G Sepharose 4 Fast Flow. The IgA of *T. truncatus* was purified by affinity chromatography using jacalin-agarose. Affinity columns using the ImmunoPure® Immobilized mannan binding protein have been demonstrated to bind mouse and human IgM. It was thus considered that mannan binding protein might prove successful as a

means of purifying T. truncatus IgM. The eluate (containing the desired immunoglobulin) obtained from the column was concentrated in a centrifugation filter and then electrophoresed on a SDS-PAGE apparatus along with molecular weight standards and commercially purified human and/or mouse immunoglobulins to confirm the efficacy of purification process.

ACCOMPLISHMENTS:

Objective 1. In the course of the project, a total of 6 fusion experiments were conducted in efforts to produce heterohybridomas. Altogether, a total of 64 putative heterohybridoma clones were obtained. It was necessary to test each of these putative heterohybridomas for production of immunoglobulin. A "sandwich" ELISA format was used as the primary screen for antibody production in the developmental heterohybridomas. The results of the initial ELISA screening tentatively identified 8 of the heterohybridoma cultures as positive for production of *T. truncatus* immunoglobulin. Unfortunately, confirmatory followup screenings two weeks after the initial screenings were negative for all clones except clone 6-5. SDS-PAGE analysis was also done using supernatants from cultures of all clones initially testing positive. With the exception of clone 6-5, SDS-PAGE analysis of the heterohybridoma supernatants also were negative. Dilutions of supernatant from clone 6-5, concentrated to contain 5mg total protein per ml, showed the presence of a protein band whose molecular weight was consistent with that of *T. truncatus* IgG. Unfortunately, during the course of efforts to subclone this heterohybridoma, the cultures ceased to produce the desired immunoglobulin.

Two additional methods were employed in efforts to confirm (for secretors) or ascertain (for non-secretors) whether the putative heterohybridomas were in fact heterohybridomas, or were instead simply CRL 1580 "revertants" that escaped the HAT selection process. In the first effort to this end, cultures of the set of putative heterohybridomas from the first fusion attempt were sent to Dr. Tracy Romano (presently on the staff of Mystic Aquarium) who screened each of the cultures for evidence of *T. truncatus* antigens on the cells. Specifically she used appropriate monoclonal antibodies to test for Class II MHC, T cell, B cell, and T helper cell surface proteins. In her tests, all heterohybridomas appeared to be negative (for expression of the *T. truncatus* markers selected), whereas positive controls (lymphocytes obtained by her from *T. truncatus*) were indeed positive. The cells thus did not express surface antigens which might be expected of dolphin/mouse heterohybridomas.

In the second approach to confirmation of the putative heterohybridomas, chromosome spreads were prepared from all cultures testing as positive for immunoglobulin production and from 5 additional, randomly selected, cultures testing negative for immunoglobulin production. Chromosome analysis was initially performed when the cultures were at passage level no greater than 5. All cultures tested initially showed the presence of chromosomes of morphology typical of *T. truncatus*, which are generally more elongated, which have a "twisted" appearance in comparison to mouse chromosomes, but in no case could it be determined that a full complement of *T. truncatus* chromosomes was present in addition to the mouse chromosomes. In fact, each cell line evaluated contained only a few (up to perhaps 10, but most with only one of two) chromosomes consistent with the appearance of *T. truncatus* chromosomes. Subsequent chromosome analysis of these lines, after 5-10 more subcultures, failed to show the presence of any putative *Tursiops* chromosomes. This loss of the "alien" chromosomes was not unexpected, as it is known to occur in hybrid cells.

Objective 2. Numerous hybridoma fusion experiments were conducted and resulted in the development of three hybridomas producing monoclonal antibodies of particular interest. One of these hybridomas produced antibodies reactive with both IgG1 (purified using Protein G affinity chromatography) and with IgG2 (purified using the thiophilic resin column). The mice from which this hybridoma was derived were immunized with IgG1; since the resulting monoclonal antibodies were reactive with both IgG1 and IgG2 (Tt gamma Hc), the antibodies are presumed to be specific to a common epitope present on both isotypes. The second hybridoma of interest was derived from mice immunized with Jacalin purified IgA, and produced monoclonal antibodies specific for the alpha heavy chain of IgA (Tt alpha Hc). Western blot assays using these two monoclonal antibodies revealed that both were specific for the respective heavy chains of the immunoglobulins (i.e. gamma heavy chains in one case, alpha heavy chains in the other). The third hybridoma of interest was derived from mice immunized with IgG1 and produced monoclonal antibodies for the light chain (Tt Lc). The initial ELISA screening was for reactivity to IgG1, and employed IgG1 as the antigen. Subsequently, a Western blot was performed to confirm isotype and peptide specificity of the monoclonal antibody. In this Western blot, IgA was also included as an antigen. The results of this assay revealed that this monoclonal antibody was actually specific for the light chains present in both IgG and IgA. After the specificity the three monoclonal antibodies had been determined, the isotype of the monoclonal antibodies was determined using a mouse monoclonal antibody isotyping strip. Based on the position of blue bands on the strip, the isotype of the monoclonal antibodies is revealed. The Tt gamma Hc monoclonal antibody was found to be mouse IgA and both the Tt alpha Hc and Tt Lc monoclonal antibodies were found to be mouse IgG1. SDS- PAGE analysis of the monoclonal antibodies showed molecular weights which were consistent with what would be expected based on the results of the mouse isotyping strips. Identification of the mouse isotype of the monoclonal antibody is important for instances where it is desirable to use the antibodies in indirect ELISA's where a labeled "second antibody (i.e. antibody specific for the appropriate mouse isotype) is employed. The information is also important for selection of an affinity chromatography method for purification and concentration of the monoclonal antibodies from hybridoma supernatants.

The monoclonal antibodies developed were used in appropriately designed ELISAs to demonstrate the capability of obtaining data on levels of IgG and IgA in serum samples and mammary gland secretion samples from various Atlantic bottlenose dolphin populations. Samples from captive dolphins were supplied by the U. S. Navy Marine Mammal Program (San Diego, CA), Anheuser-Busch SeaWorld Parks (San Diego, CA; San Antonio, TX,; Orlando, FL), Dolphin Encounters (Bahamas), and Marine Life Oceanarium (Gulfport, MS). Samples from a wild population were supplied by Dr. Randy Wells of Mote Marine Lab (Sarasota, FL) from a group of dolphins living in the Sarasota, FL area. 15 individual animals from each institution were represented in each assay. A competitive ELISA was developed to measure immunoglobulin levels. After the assay, concentrations of the immunoglobulin were plotted with a semi-log scale on Sigma Plot graphing program. A linear regression analysis was performed to produce an equation that would yield the concentrations of the immunoglobulin in the diluted serum samples. The mean of the replicates was calculated using bootstrapping, a statistical analysis procedure. An absorbance value that falls in the range of the standard curve was inserted into the regression line equation to yield a concentration which is then multiplied by the dilution factor to give a concentration in 1 per ml.

A scatter plot showing the concentrations of IgG are was graphed for 100 *T, truncatus*

serum samples representing 69 individuals. A similar scatter plot showing the concentrations of IgA for 50 *T. truncatus* serum samples representing 43 individuals was also graphed. Data obtained relative to IgG concentrations in 126 Navy *T. truncatus* serum samples taken in years 2000-2003. The samples represent 54 individual animals. For 38 of the animals two or more samples were available for assay. The range of IgG concentrations was 0.5 to 13.6 mg/ml with an average concentration for the 54 animals of 5.9 mg/ml with a standard deviation of 2.5. If we consider the normal range to be equal to the mean plus or minus one standard deviation, then the normal range would be 3.5 to 8.5 mg/ml. Using this range there were 11 animals that fell below this "normal" range and 8 animals that were above it. Various trends (e.g. increasing concentrations, decreasing concentrations) can be seen within the results for individuals with multiple samples; however, there are still too few results to make any definitive interpretations especially without further information regarding the health and history of the animals.

Relative concentrations of *T. truncatus* IgG and IgA were determined in a number of samples of mammary gland secretions. Concentrations were determined by ELISA using the Tt gamma Hc and Tt alpha Hc, and were compared to published levels of IgG and IgA in several other species. As expected, the dolphin has a greater amount of IgG being secreted then IgA. This is the opposite of the situation in humans which secrete mostly IgA, but more similar to the artiodactyl species which secrete most IgG. The relative amount of IgG/IgA secreted by *T. trucatus* is in fact quite similar to the relative amounts reported in cattle. These data are not unexpected given the generally accepted phylogenetic relationship between artiodactyls and cetaceans.

Concentrations of IgG and IgA were compared in relation to captive (open ocean and closed systems) versus wild *T. truncatus*. There is a significant difference between concentrations in animals from captive/closed system versus captive/open ocean and wild environments. The p values of wild versus captive/open ocean ($p<0.005$) and wild versus captive/closed system ($p<0.00002$) are much smaller than the p value of captive/open ocean versus captive/closed system ($p<0.026$). These differences presumably reflect the results of life in a wild environment with uncontrolled exposure to potentially numerous immunogens (including various pathogens) versus life in captive environment where environmental exposure to immunogens is limited by restricted movement of the animals or by extensive control of the environment (e.g. by filtration/treatment of the water).

Objective 3. Purification of IgG. Analysis of the SDS-PAGE results indicated that the putative IgG was composed of two peptides, one having a molecular weight of 51-54 kDa (consistent the molecular weight of IgG heavy chains from many species) and another having a molecular weight of 29.5 kDa (consistent with the molecular weight of light chains from many species). Of interest was the observation that after Protein G affinity chromatography, followed by Jacalin affinity chromatography, then by mannan-binding protein binding chromatography, there remained a protein present which had peptide subunits consistent with those of IgG. The existence of at least one additional isotype of IgG which for which Protein G did not have affinity was therefore postulated. The existence of at least one such additional isotype of IgG is consistent with findings of multiple IgG isotypes in most mammalian species investigated. As indicated in the section of this report describing efforts to purify *T. truncatus* IgM (see below, Purification of IgM)), one of the methods evaluated for IgM purification, specifically T-Gel™ chromatography, was found to be useful for purification of an apparent second isotype of IgG.

The two forms (putative isotypes) of IgG have been tentatively assigned the designations IgG1 (purified by Protein G chromatography) and IgG2 (purified by T-Gel™ chromatography)

Purification of IgA. Analysis of the purified proteins on the SDS-PAGE gels indicated a molecular weight of the putative alpha heavy chain of 63.3 kDa (consistent with the alpha chain molecular weight of many species). The molecular weight of the dolphin light chains obtained from the putative IgA was 29.5 kDa, identical to that of the light chains associated with the dolphin IgG, a result that would be expected.

Purification of IgM. Initially, mannan binding protein chromatography was employed in the purification of IgM. The results of SDS-PAGE analysis indicated similar proteins in both the pass through volume and the subsequently eluted fluid, although the concentrations of proteins in the eluted volume were much lower, based on intensity of staining of bands in the respective volumes. After several efforts, it was concluded that the mannan binding protein matrix did not provide an effective means for purification of IgM from *T. truncatus* serum. Based upon this conclusion, another affinity chromatography method for purification of IgM was evaluated. The T-Gel™ Adsorbent column is based on thiophilic adsorption chromatography. The T-Gel column was evaluated for ability to bind to any *T. truncatus* immunoglobulins remaining in serum that had been exhausted of IgG from Protein G binding and IgA from Jacalin binding. SDS-PAGE and Grabar-Williams analysis of the pass through fraction was used to demonstrate whether or not IgG was present(by banding pattern, size, and reactivity to antisera against *T. truncatus* IgG). An SDS-PAGE analysis of the elution fraction of a typical application of the T-Gel™ column and the pass through and absorbance peaks of a typical application of the T-Gel™ column showed a banding pattern that resembled IgG rather than IgM (based on molecular weight of the heavy chain fraction). A Grabar-Williams immunoelectrophoresis was performed on the pass through and elution fractions. The results of this assay also indicated that the properties of the protein obtained from the T-Gel™ were consistent with its being IgG and not IgM. Further confirmation that the T-Gel was removing an IgG subclass (provisionally designated IgG2) different from that obtained using the Protein G column (provisionally designated IgG1) was provided by the observation that when the elution peak of the T-Gel column (containing the immunoglobulin of interest) was applied to the Protein G column, none of the protein bound to the column.

Since none of the affinity chromatography methods available proved useful for purification of IgM, size exclusion chromatography was attempted using gel filtration column. The first protein peaks obtained using these columns contained small amounts of a high molecular weight protein consistent with IgM. The relatively small concentration of IgM in *T. truncatus* serum and the relatively small volumes of serum which were available during the course of the project were the major barriers to employing size exclusion chromatography for purification of sufficient amounts of purified IgM for production of monoclonal antibodies and for use in preparation of standards for use in quantitative assays measuring IgM levels.

CONCLUSIONS: Heterohybridomas created by fusion was successful, however, they do not appear to be stable with respect to the retention of the "alien" immunoglobulin genes. Purification of the three major classes (IgG, IgA, and IgM) of immunoglobulin was successful. Two putative subclasses of IgG (IgG1 and IgG2) were also purified. Three hybridomas were created using the purified immunoglobulin. They were specific for *T. truncatus* gamma heavy chain, alpha heavy chain, and the light chain. The hybridomas created against the gamma and

alpha heavy chains were successfully used in an ELISA format to determine immunoglobulin levels in serum and other secretions from T. truncatus.

SIGNIFICANCE: Development of easily implemented quantitative assays for immunoglobulin isotype concentrations in dolphins provides an immune health parameter assessment that can be applied on a regular basis to establish baseline levels for individual animals in captivity, and which can be used to monitor animals before, during, and after missions or field exercises. Immunoglobulin levels as an immune health parameter should be used in conjunction with other parameters, measuring, for example specific cellular immunity (moderated by T-cells) and non-specific immune functions. Development of heterohybridomas will provide a valuable tool for in vitro production of large amounts of cetacean immunoglobulins as an alternative to gene cloning techniques. Heterohybridoma production also has the potential to permit identification of minor immunoglobulin isotypes that might be overlooked by protocols requiring purification of significant amounts of immunoglobulin.

PUBLICATIONS and ABSTRACTS (for total period of grant):

1. Lilly, N.A., R.A. Patterson, and B.L. Middlebrooks. (2002) Development of a monoclonal antibody against *Tursiops truncatus* IgG for use as a reagent in a diagnostic ELISA. Proceedings of the 33rd Annual International Association for Aquatic Animal Medicine Conference and Workshop, p 8.
2. Patterson, R.A. and B. L. Middlebrooks. (2002) Methods for purification and study of cetacean immunoglobulins. *in* Cell and Molecular Biology of Marine Mammals , Krieger Publishing Company, Melbourne, Florida. p 193-204.
3. Middlebrooks, B. L., J,C, Jones, and R. A. Patterson. (2002) Application of ELISA methodology for detection of *Erysipelothrix rhusiopathiae* antibody titers in cetaceans. *in* Cell and Molecular Biology of Marine Mammals, Krieger Publishing Company, Melbourne, Florida. p 245-252.
4. Lilly, N.A., R.A. Patterson, and B. L. Middlebrooks. (2003) Development of monoclonal antibodies against *Tursiops truncatus* (Atlantic bottlenose dolphin) immunoglobulin isotypes. Programme and Abstracts of the 9th International congress of the International Society for Developmental and Comparative Immunology, p 124.
5. Patterson, R.A., N.A. Lilly, and B..L. Middlebrooks. (2004) IgG and IgA concentrations determined in serum and milk from Atlantic bottlenose dolphins (*Tursiops truncatus*) with monoclonal antibodies specific for the immunoglobulin isotypes. Proceedings of the 35th Annual International Association for Aquatic Animal Medicine Conference and Workshop, p 134.
6. Garrick, R.A., B.R. Woodin, J.Y. Wilson, B.L. Middlebrooks, and J.J. Stegeman. (2006) Cytochrome P4501A is induced in endothelial lines from the kidney and lung of the Atlantic bottlenose dolphin , *Tursiops truncatus*. Aquatic Toxicology 76: 295-305.